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IMMEDIATE COMMUNICATION

Two variants in *Ankyrin 3* (*ANK3*) are independent genetic risk factors for bipolar disorder

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Two recent reports have highlighted *ANK3* as a susceptibility gene for bipolar disorder (BD). We first reported association between BD and the *ANK3* marker rs9804190 in a genome-wide association study (GWAS) of two independent samples (Baum *et al*., 2008). Subsequently, a meta-analysis of GWAS data based on samples from the US and the UK reported association with a different *ANK3* marker, rs10994336 (Ferreira *et al*., 2008). The markers lie about 340 kb apart in the gene. Here, we test both markers in additional samples and characterize the contribution of each marker to BD risk. Our previously reported findings at rs9804190, which had been based on DNA pooling, were confirmed by individual genotyping in the National Institute of Mental Health (NIMH) waves 1–4 ($P = 0.05$; odds ratio (OR) = 1.24) and German ($P = 0.0006$; OR = 1.34) samples. This association was replicated in an independent US sample known as NIMH wave 5 (466 cases, 212 controls; $P = 0.017$; OR = 1.38). A random-effects meta-analysis of all three samples was significant ($P = 3 \times 10^{-6}$; OR = 1.32), with no heterogeneity. Individual genotyping of rs10994336 revealed a significant association in the German sample ($P = 0.0001$; OR = 1.70), and similar ORs in the NIMH 1–4 and NIMH 5 samples that were not significant at the $P < 0.05$ level. Meta-analysis of all three samples supported an association with rs10994336 ($P = 1.7 \times 10^{-5}$; OR = 1.54), again with no heterogeneity. There was little linkage disequilibrium between the two markers. Further analysis suggested that each marker contributed independently to BD, with no significant marker × marker interaction. Our findings strongly support *ANK3* as a BD susceptibility gene and suggest true allelic heterogeneity.
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Introduction

The gene *ANK3* encodes ankyrin-G, a large protein whose neural-specific isoforms, localized at the axonal initial segment and nodes of Ranvier, may help maintain ion channels and cell adhesion molecules.[1] *ANK3* was first implicated in the etiology of bipolar disorder (BD) by a genome-wide association study carried out in a US discovery and a German replication sample, totaling about 1000 cases and controls.[2] A single nucleotide polymorphism (SNP) in an intron of *ANK3* (rs9804190) was one of 88 that met criteria for replication in that study. Recently, Ferreira *et al.*[3] reported that several SNPs spanning a ∼145 kb region in and around *ANK3* were associated with BD in a meta-analysis of some 4000 cases and 6000 controls from the United States and the British Isles. Among these, rs10994336, whose genotypes were imputed, showed the strongest association signal.

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The report of Ferreira *et al.*[3] prompted us to revisit our earlier findings. In the present study, we address three questions: (1) Do our published results, which were based on pooled DNA, hold up after individual genotyping? (2) Do the association signals at rs9804190 and rs10994336 replicate in independent samples? (3) Do these two *ANK3* markers, separated by approximately 340 kb, act independently to influence risk for BD?

Methods

Samples

German case–case control sample. This sample was used in our previous GWAS analysis. Detailed ascertainment and phenotype information was presented in Baum *et al.*[2] For the present study, 745 cases (401 men, 344 women; mean age 44 ± 13) with *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (DSM-IV)-defined bipolar 1 disorder from consecutive hospital admissions and 830 population-based control individuals (447 men, 383 women; mean age 49 ± 16) were available. The study protocol was approved by the Ethics Committees of the Faculties of Medicine of the Universities of Bonn and Heidelberg.

NIMH 1–4 case–control sample. This sample was used in our previous GWAS analysis.[2] Cases were drawn from waves 1 through 4 of the National Institute of Mental Health (NIMH) Genetics Initiative (http://nimhgenetics.org), a sample of multiplex families ascertained through sibling pairs with bipolar 1 or schizoaffective BD. One proband per family was selected for study, as detailed in Baum *et al.*[2] The control sample was also ascertained by the NIMH Genetics Initiative with the help of a marketing firm. For the present analysis, 457 cases (166 men, 291 women; mean age 41 ± 11) with bipolar I disorder ($n = 451$) or schizoaffective-bipolar disorder (SABP; $n = 6$), and 562 screened control individuals (315 men, 247 women; mean age 57 ± 17) were available. All subjects were of European descent and were collected under protocols approved by the local Institutional Review Boards.

NIMH 5 case–control sample. This constitutes an additional sample that has previously not been used in our studies. Both cases and controls were recruited within the framework of wave 5 of the NIMH Genetics Initiative. The ascertainment scheme allowed for the inclusion of unrelated subjects with a diagnosis of DSM-IV bipolar I disorder or SABP, without regard to family history. Controls consisted of volunteers collected by the NIMH Genetics Initiative who were not available at the time of our initial study. The sample included 466 cases (237 men, 229 women; mean age 43 ± 13) with DSM-IV-defined bipolar I disorder ($n = 438$) and SABP ($n = 28$), and 212 control individuals (119 men, 93 women; mean age 51 ± 17). All subjects were of European descent and were collected under protocols approved by the local Institutional Review Boards.

Markers and genotyping

In the German, NIMH 1–4, and NIMH 5 samples, we studied the following *ANK3* markers: rs9804190 (C/T) located in intron 36 between exons 36 and 37 (bp position 61509837) and rs10994336 (C/T) located 30 kb downstream of *ANK3* (bp position 61849818). Location assignment is based on RefSeq NM_020987 of the UCSC Genome Browser (http://genome.ucsc.edu).

Single nucleotide polymorphisms were individually genotyped using a modification of the 5′ nuclease (TaqMan) assay, scored on a fluorescent plate reader by use of a clustering algorithm (kindly provided by Sam Chen, Virginia Commonwealth University). Genotyping accuracy was ensured by genotyping of duplicate samples on each plate.

Analysis

PLINK (version 1.03) (4; http://pngu.mgh.harvard.edu/~purcell/plink/) was used for primary association testing and to assess deviation from Hardy–Weinberg equilibrium. Analyses were performed for each sample individually. Linkage disequilibrium (LD) between the two SNPs was calculated using the UNPHASED 3.08 software.[5]

A Mantel–Haenszel random effects meta-analysis, implemented in *Review Manager*,[6] was used to assess association between BD and the two *ANK3* SNPs in multiple samples. Heterogeneity between the samples was assessed using the I^2 statistic.[7,8]

To explore further the individual contribution of each SNP to BD susceptibility in the combined German, NIMH 1–4, and NIMH 5 samples, we tested for independent effects of each SNP and potential interaction between them with PLINK.[4] Independence of the two SNPs was tested using the *logistic* and *condition* functions. We tested the effect of rs9804190 while adding the allelic dosage for rs10994336 as a covariate, and vice versa. Interaction was tested using the *interaction* function, which is identical to the interaction term in a standard logistic regression analysis.

Results

Genotyping call rates ranged from 98% (NIMH 1–4 controls) to 100% (NIMH 5 cases) for rs9804190, and from 93% (NIMH 1–4 cases) to 96% (German cases) for rs10994336. Both SNPs were within Hardy–Weinberg expectations ($P > 0.05$) in all samples.

rs9804190 in the German, NIMH 1–4, and NIMH 5 samples

The association between rs9804190 and BD that we observed in our earlier study[2] held up after individual genotyping in the German and NIMH 1–4 samples. The C allele was consistently more frequent in cases than in controls (German sample: $P = 0.0006$, odds ratio (OR) = 1.34; NIMH 1–4 sample: P = 0.05, OR = 1.24). In the independent NIMH 5 sample, we also observed a significant excess of the C allele in cases (P = 0.017, OR = 1.38). The meta-analysis supported the observations in the individual samples (P = 0.000003; OR = 1.32), with an I^2 heterogeneity score of 0 (Table 1; Figure 1).

rs10994336 in the in the German, NIMH 1–4, and NIMH 5 samples

The SNP rs10994336, which was imputed in the Ferreira *et al.*[3] study, was also associated with BD in the German dataset. The T allele was more frequent in cases than in controls (P = 0.0006, OR = 1.70). No significant differences in allele frequencies were observed in either of the two smaller NIMH samples (P = 0.112 in NIMH 1–4 and P = 0.241 in NIMH 5), but an overall association was supported by meta-analysis of the combined samples (P = 0.0000170, OR = 1.54, I^2 = 0; Table 1; Figure 1).

Dissecting the relationship between rs9804190 and rs10994336

There was no detectable LD between the two markers. In the combined German, NIMH 1–4, and NIMH 5 samples (n = 3272), the r^2-value was 0.003 in controls and 0.002 in cases. Each SNP was a significant, independent predictor of affection status (t = 3.987, P = 0.00007 for rs9804190, conditioned on rs10994336; t = 3.352, P = 0.0008 for rs10994336, conditioned on rs9804190). No evidence of interaction between rs9804190 and rs10994336 was detected (P = 0.93).

Discussion

These results, in a combined set of 1668 cases and 1604 controls, provide strong support for association of two markers in *ANK3* with BD across several independent samples, comprising populations from the United States and Germany. Both rs9804190 and rs10994336 were significantly and consistently associated with BD in most of the samples we studied, and both yielded significant overall association by meta-analysis. Although rs10994336 was not significantly associated with BD in either NIMH sample, the consistency of the odds ratios suggests that the lack of significance in the NIMH samples is attributable to low statistical power, rather than true heterogeneity. These findings demonstrate that replicable association findings are attainable in BD, with sufficient sample sizes, but universal association of individual SNPs across all samples may be an unrealistic goal.

The NIMH control samples we used overlap with those in the Ferreira *et al.*[3] study. However, the largest effects we observed were in the German sample, which was completely independent from that of Ferreira *et al.*[3] Thus, spurious association because of atypical allele frequencies in the NIMH controls seems highly unlikely.

Table 1 Results from the single marker case–control association analyses and the meta-analysis

Marker	*German*			*NIMH 1-4*			*NIMH 5*			*Meta-analysis*
	Cases (N = *745*)	*Controls* (N = *830*)	P-*value* (*OR*)[a]	*Cases* (N = *457*)	*Controls* (N = *562*)	P-*value* (*OR*)[a]	*Cases* (N = *466*)	*Controls* (N = *212*)	P-*value* (*OR*)[a]	P-*value* (*OR*)
rs9804190 (alleles: C/T)	0.20 (T)	0.25 (T)	0.0006 (1.34)	0.20 (T)	0.23 (T)	0.050 (1.24)	0.21(T)	0.27 (T)	0.017 (1.38)	3×10^{-6} (1.32)[b]
rs10994336 (alleles: C/T)	0.10 (T)	0.06 (T)	0.0001 (1.70)	0.06 (T)	0.04 (T)	0.112 (1.39)	0.08 (T)	0.06 (T)	0.241 (1.33)	1.7×10^{-5} (1.54)[b]

Abbreviations: NIMH, National Institute of Mental Health; OR, odds ratio.
[a]To ease comparisons, ORs were calculated with respect to the T allele of each SNP.
[b]Test for heterogeneity: I^2 = 0%.

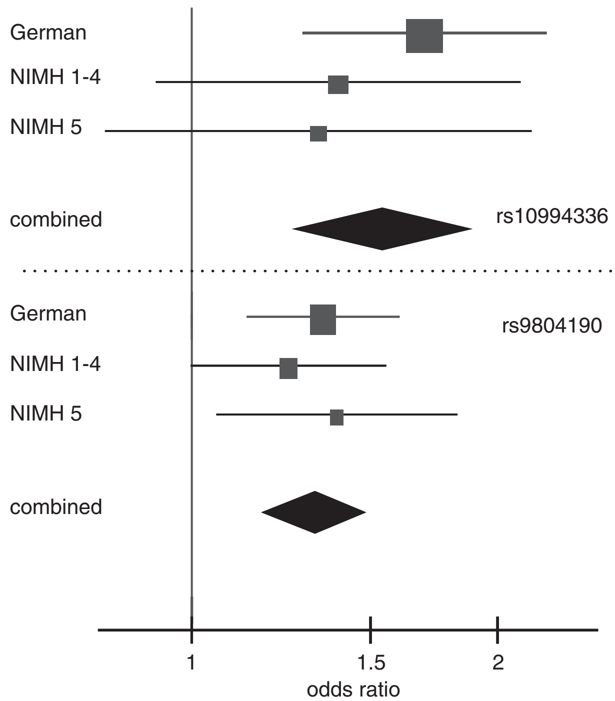

Figure 1 Forest plots of odds ratios and their 95% confidence intervals for the individual samples and the meta-analysis.

As rs9804190 was one of 88 SNPs initially implicated in an earlier GWAS,[2] any one of which may be the subject of future studies, the present results may need to be corrected for multiple testing. The meta-analysis *P*-value of 3×10^{-6} remains significant at the $P<0.01$ level, whether corrected by a factor of 88 or by the factor of 1877 we adopted in the earlier study.[2]

Our analyses suggest that each of the two *ANK3* markers we studied is an independent risk factor for BD. The two markers are located over 340 kb apart and there is little detectable LD between them. Furthermore, each marker was a significant, independent predictor in a logistic regression analysis that included both markers. We could not detect any evidence of marker × marker interaction. Our findings may be attributable to true allelic heterogeneity, where more than one functional variant in the same locus contributes to disease risk. Allelic heterogeneity is the rule for Mendelian disorders, but to our knowledge has not yet been demonstrated for a common disease such as BD.

Taken together, the findings of Baum *et al.*,[2] Ferreira *et al.*[3] and the present study strongly suggest that *ANK3* is one of the many genes that play a role in the etiology of BD. Other genes have been implicated in previous meta-analyses.[3,9] It seems likely that additional genes will be identified as sample sizes grow and complementary approaches—such as those focused on rare alleles and subphenotypes—are pursued.

Although BD is a polygenic disease, the identification of individual risk genes may still reveal important aspects of etiology. Ankyrins belong to a family of proteins that connect integral membrane proteins to the spectrin–actin cytoskeleton. Animal studies have shown that ANK3 is essential for both normal clustering of voltage-gated sodium channels at axon initial segments and normal action potential firing.[1,10,11] These are very basic mechanisms of brain function, only distantly related to the widely held theories that focus on neurotransmitters or other signaling pathways.

Our finding that two SNPs act independently within the same gene to influence risk underscores the importance of scrutinizing a broad range of genetic variation in a potential susceptibility gene highlighted by GWAS. Such scrutiny should be a goal of future studies of *ANK3*. Additional important markers may yet to be found, but the regions near rs9804190 and rs10994336 are logical foci for future work aimed at defining the functional variants that account for these association findings.

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